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Amplification of Ki-*ras* and elevation of MAP kinase activity during mammary tumor progression in C3(1)/SV40 Tag transgenic mice

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We have previously documented that transgenic mice expressing SV40 Tag regulated by the rat prostatic steroid-binding protein C3(1) 5′-flanking region display multistage mammary tumorigenesis. To delineate genetic changes associated with mammary tumor progression, comparative genomic hybridization (CGH) was performed. CGH revealed a consistent gain of the telomeric region of chromosome 6. This region contains the Ki-*ras* proto-oncogene. Analyses of genomic DNA by Southern blot demonstrated up to 40-fold amplification of the Ki-*ras* gene. Ki-*ras* amplification was detected in 12, 46 and 68% of tumors from 4, 5 and 6 month old mice, respectively, whereas no amplifications were found in any preneoplastic mammary tissues. Tumors bearing Ki-*ras* gene amplification exhibited high levels of Ki-*ras* RNA and protein. The over-expressed Ki-Ras protein in these tumors appeared functionally active as indicated by the elevated MAP kinase activity. These data demonstrate that while Ki-*ras* amplification might not be an early event, there is a strong association between Ki-*ras* amplification and over-expression and mammary tumor progression in this model. This study also shows that CGH is a powerful and useful technique for identifying chromosomal copy number changes during tumor progression, and that this model may provide a predictable *in vivo* system for studying gene amplification.

Keywords: CGH; Ki-*ras*; gene amplification; MAP kinase; mammary gland tumor; transgenic mice; Ras-bound GTP and GDP

Introduction

Breast cancer is the most common malignancy in US women, representing about 25% of all female cancer. In Western Europe and the United States, approximately one in every 12 women develop breast cancer (Wooster *et al*., 1995). As has been shown for the development of most cancers, breast cancer may arise through a multistep process involving multiple genetic alterations (Christofori and Hanahan, 1994; Devilee and Cornelisse, 1994; Fearon and Vogelstein, 1990). Alterations in oncogene and tumor suppressor gene function through gene amplification, mutation, deletion, chromosomal rearrangement or translocation are among the common and important mechanisms (Jones *et al*., 1995). The oncogenic targets of some of the amplified regions have been relatively well studied and protooncogenes identified, whereas the genes involved in other regions remain undefined and may include many candidate oncogenes and tumor suppressor genes. Even though a large number of genetic alterations have been identified in human breast cancer, the genetic events underlying initiation and progression are still not clear and are difficult to study in individual patients.

We have used our previously described C3(1)/Tag transgenic mouse model of mammary cancer (Maroulakou *et al*., 1994) to look for genetic alterations during mammary tumor progression. Female transgenic mice expressing SV40 Tag under the regulatory control of the rat prostatic steroid binding protein C3(1) 5′ flanking region display a multistage progression of mammary cancer ranging from normal (tissues prior to 2 months of age), to preneoplastic hyperplastic lesions beginning at 2 months of age, to subsequent nodular hyperplasia at about 3 months of age and finally to the development of adenocarcinoma in 100% of animals after 4 months of age (Shibata *et al*., 1996a). The initiation of tumor formation appears to occur at an early age. However, additional genetic events seem necessary for tumor progression. Because the course of development from premalignant lesions to carcinoma in this model is well defined, it is a valuable system for dissecting genetic alterations during the multistage progression of mammary tumorigenesis.

In this study, we screened for chromosomal copy number changes associated with mammary tumor progression using comparative genomic hybridization (CGH). CGH revealed a consistent amplification of the telomeric portion of chromosome 6 which contains the Ki-*ras* protooncogene. Further molecular analyses were conducted to determine the relationships between Ki-*ras* gene amplification and mammary gland tumor progression in the C3(1)/SV40 Tag transgenic mice.

Results

Amplification of chromosome 6 telomeric region

To screen for chromosomal copy number changes, CGH was conducted in mammary tumors from C3(1)/SV40 Tag transgenic mice as previously described (Ried *et al*., 1995). In this application, equal amounts of Bio-16-dUTP-labeled tumor DNA and digoxigenin-

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11-dUTP-labeled normal DNA were simultaneously hybridized to normal mouse metaphase spreads. The Biotin-labeled tumor DNA was detected by green-fluorescing avidin-FITC and digoxigenin-labeled normal DNA was detected by red fluorescing rhodamine-antidigoxigenin (Figure 1). Although enhanced tumor specific green fluorescence was observed occasionally in chromosome 1, 11 and X, consistent peak green fluorescence intensities was observed in the telomeric region of chromosome 6 (Figure 1c), indicating a significant amplification of this region in the mammary tumors of C3(1)/SV40 Tag transgenic mice.

Amplification of Ki-ras *gene*

The telomeric region of mouse chromosome 6 contains a known proto-oncogene, Ki-*ras*. Southern blot analysis was, therefore, performed to determine whether the Ki-*ras* gene was amplified. Using a ^{32}P-labeled 6.8 kb mouse Ki-*ras* genomic fragment as probe, three bands of 2.3, 4.5 and 4.9 kb were observed. As predicted from the CGH results, the Ki-*ras* gene was confirmed to be amplified in many C3(1)/SV40 Tag mammary tumors. Figure 2a is a representative Southern blot of tumors from 4 month old transgenic mice. When compared with normal mammary tissues (Figure 2a, lanes 1 and 2), several tumors (Figure 2a, lanes 6 and 10) showed a dramatic amplification of the Ki-*ras* gene. To quantitate the levels of amplification by Southern blot analysis, we determined the Ki-*ras* gene dosage by comparing the levels of the Ki-*ras* signal intensity in mammary tumors with those in the wild-type mammary tissues, after normalizing for actin levels (Figure 2b). The levels of amplification were approximately 23–26-fold higher than the controls (Figure 2b) and up to a 40-fold

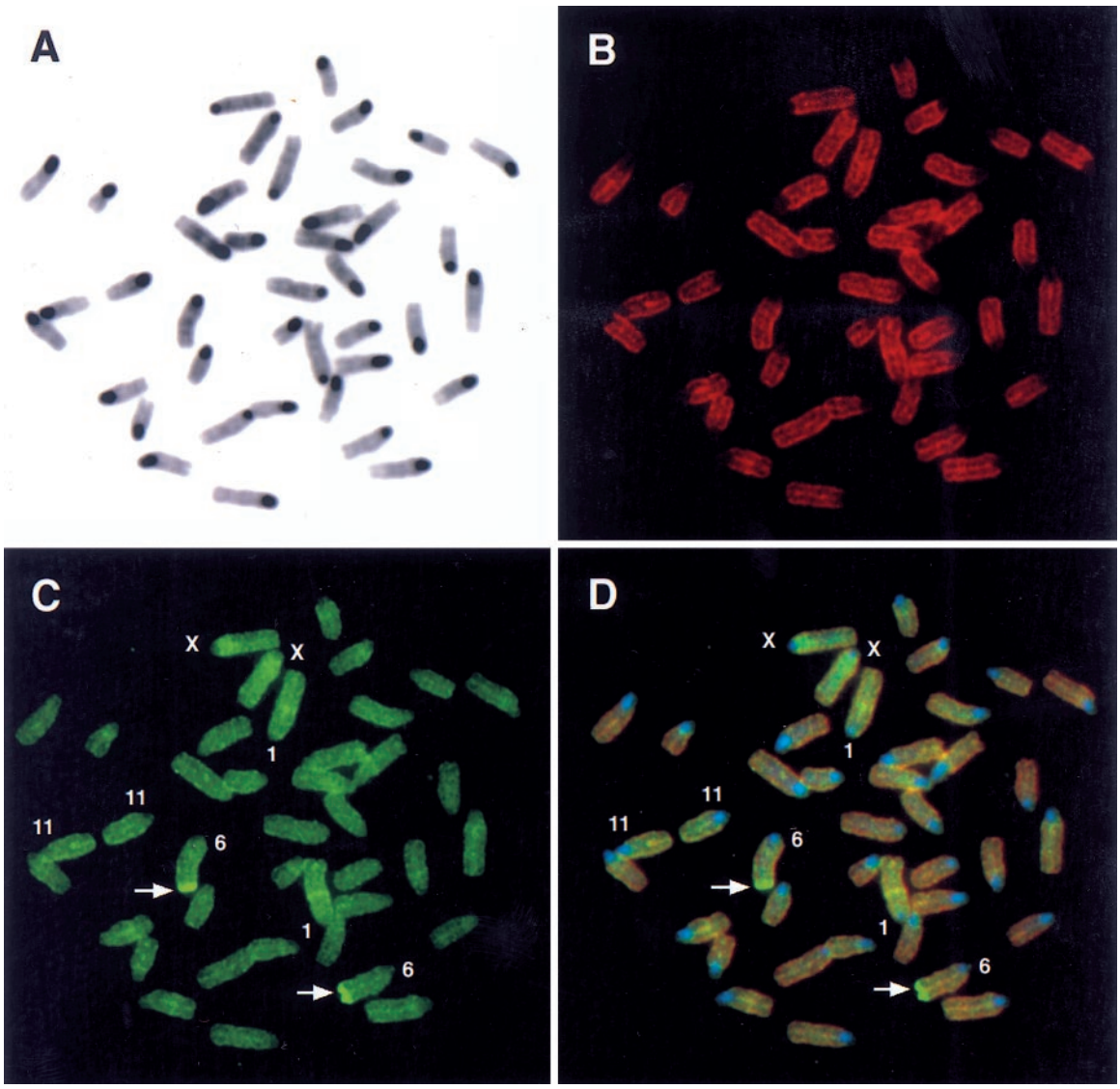

Figure 1 Representative CGH analysis of mammary tumors of C3(1)/SV40 Tag transgenic mice. (**a**) DAPI-banding of normal mouse metaphase chromosomes. The inverted image serves for chromosome identification. (**b**) Visualization of the rhodamine labeled reference genome. Note the homogeneous labeling of all mouse chromosomes. (**c**) Visualization of the fluorescein labeled tumor genome. Note that chromosome 1, 11, X reveal increased intensity in the tumor specific fluorescence, indicating a gain of these chromosome. However, consistent peak fluorescence intensities were observed in the telomeric region of mouse chromosome 6 (arrows). (**d**) Composite display of images **a**, **b**, **c**

amplification has been observed. Lack of Ki-*ras* amplification in the tail DNAs (data not shown) confirmed that the Ki-*ras* amplification was specific to the tumors and was not a generalized somatic event. The same blots were re-hybridized with Met, a protooncogene from an unrelated region of chromosome 6. No significant amplification of Met was observed (Figure 2a), which was consistent with the CGH results that DNA sequence gain was confined to the distal end of chromosome 6. Some other genes commonly amplified in breast cancer such as the c-*myc* gene of mouse chromosome 15 and ErbB2/neu/HER2 gene of mouse chromosome 11 were also analysed and no amplification of these genes was detected (data not shown).

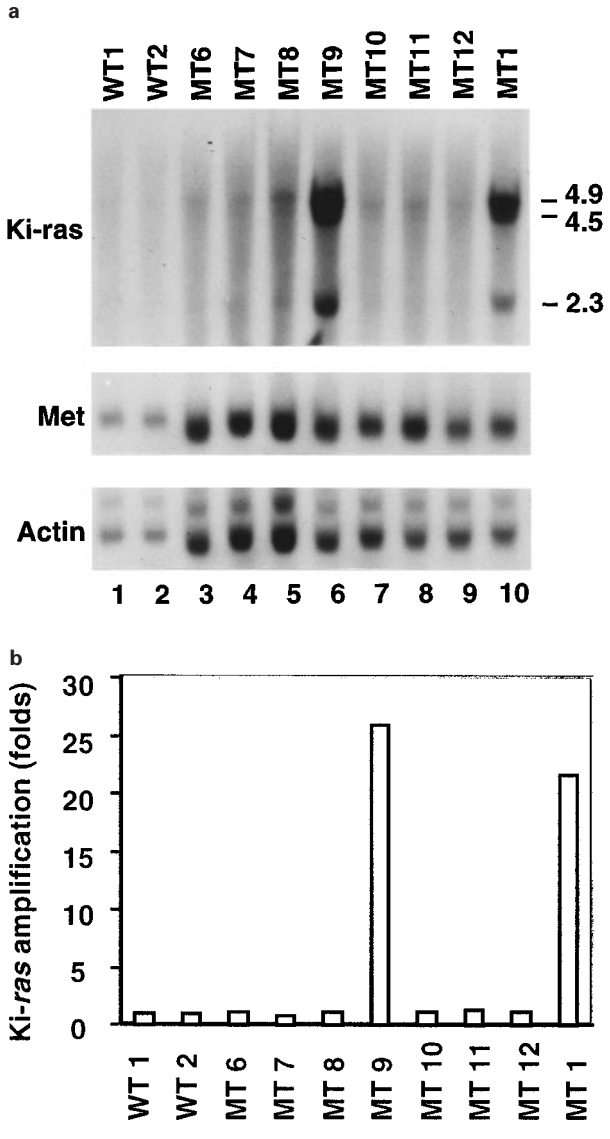

Figure 2 Representative Southern blot analyses of Ki-*ras* in mammary tumors of C3(1)/SV40 Tag transgenic mice. (**a**) 20 μg of genomic DNA was digested with *Xba*I and hybridized with indicated probes as described in Material and methods. Lanes 1 and 2, mammary tissues from wild-type FVB/N mice (WT); lanes 3–8, mammary tumors (MT) of 4 month old SV40/Tag transgenic mice. Representative bands of β-actin and Met are also shown. (**b**) Quantitation of Ki-*ras* amplification derived from Ki-*ras* Southern analysis in **a**. Results are expressed as the ratio of Ki-*ras* intensity in tumors over that in WT mammary tissue after normalizing for actin where wild-type values equal 1.0

Ki-ras *amplification is associated with mammary tumor progression*

To determine the importance of Ki-*ras* amplification and its association with mammary tumor progression in this model, mammary tissues and mammary tumors were isolated from multiple transgenic mice at 3, 4, 5 and 6 months of age respectively, representing the stages from hyperplasia to invasive carcinoma. Ki-*ras* amplification in these tissues and tumors was examined by Southern analyses. No Ki-*ras* amplification was detected in preneoplastic mammary tissues from 3 and 4 month old transgenic mice (data not shown). The rate of Ki-*ras* amplifiction was relatively low in the early stage of tumor development. As shown in Figure 3, only about 12.5% of mammary gland tumors isolated from 4 month old transgenic mice had Ki-*ras* amplifications. However, as mice aged and tumors progressed, Ki-*ras* amplification became very common. Ki-*ras* amplification was detected in 50% of tumors from 5 month old animals and 68% of tumors from 6 month old transgenic mice. These data strongly suggest that Ki-*ras* amplification is a late event associated with tumor progression.

Over-expression of Ki-ras

The expression of Ki-*ras* in C3(1)/SV40 Tag mammary tumors was examined by Northern and Western blot analyses and representative results are shown in Figures 4 and 5. Normal mammary tissues expressed two Ki-*ras* mRNA transcripts of 2.0 and 5.0 kb (Figure 4, lanes 1 and 2). Ki-*ras* mRNA levels were slightly increased in mammary tumors without Ki-*ras* gene amplification (Figure 4, lanes 3–5 and 7–9), but dramatically elevated in mammary tumors with Ki-*ras*

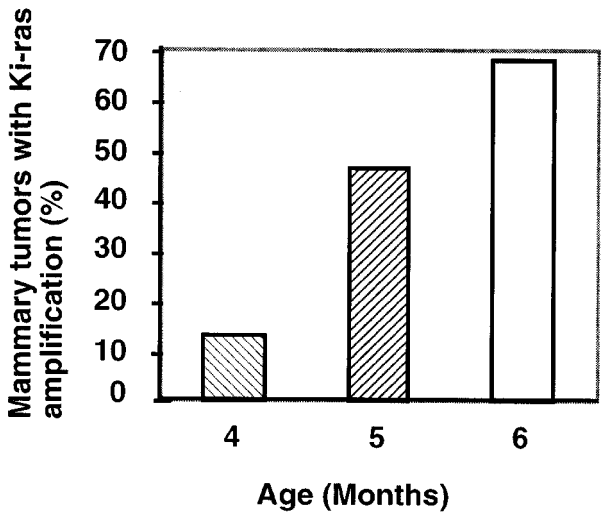

Figure 3 Levels of Ki-*ras* amplification in SV40/Tag mammary tumors as a function of age: Ki-*ras* amplification was examined in eight tumors isolated from 4 month old mice, 13 tumors from 5 month old mice and 22 tumors from 6 month old transgenic mice. Twenty hyperplastic mammary tissues from 3–4 month old transgenic mice were also analysed, but no amplification was detected (data not shown)

gene amplification (Figure 4, lane 6). Similarly, the 21 kD Ki-Ras protein (measured by Western blot using the Ki-Ras specific antibody F234) was detectable in normal mammary tissues (Figure 5, lanes 1–3), mildly elevated in mammary tumors without Ki-*ras* amplification (Figure 5, lanes 5–7)) and significantly overexpressed in tumors with Ki-*ras* amplification (Figure 5, lanes 4, 8 and 9). The elevated levels of both Ki-*ras* mRNA and protein correlated well with the degree of gene amplification.

Ras-bound GTP and GDP

To determine the functional activity of the overexpressed Ki-Ras protein, the absolute amounts of Ras-bound GTP and GDP were measured. Because normal mammary tissue contained more fat cells than the malignant samples, the normal samples had relatively less protein than the malignant ones; because some of the malignant samples were aneuploid they had relatively more DNA than the normal tissue. To eliminate these confounding variables, the data are expressed per wet weight of the sample. However, qualitatively similar results were obtained when the data are expressed per mg protein or per ug DNA (data not shown). In Table 1, the results of three representative samples from each of the three types of tissues (normal mammary gland tissue, WT; tumors without Ki-*ras* amplification, MT− and tumors with Ki-*ras* amplification, MT+) are shown. Surprisingly, all three types of tissue had approximately the same amount of Ras in the active GTP-bound state (Table 1, column 2). However, the tumors without Ki-*ras* amplification showed an approximate sevenfold increase in Ras-bound GDP when compared to Ras-bound GDP in the normal tissue and the tumors with Ki-*ras* amplification showed an average of almost 60-fold increase in Ras-bound GDP (Table 1, column 3). Thus, the increased Ki-*ras* that was expressed in the tumors was in the inactive GDP-bound state. Because of the large increase in Ras-bound GDP in the tumors without any change in Ras-bound GTP, the percent of Ras molecules in the GTP-bound state was actually reduced in the tumors compared to normal mammary tissue (Table 1, column 5).

The large increase in Ras protein (which is the sum of Ras-bound GTP plus Ras-bound GDP, Table 1, column 4) in the tumors with Ki-*ras* amplification without any change in Ras-bound GTP, suggests that there may be some regulatory mechanism in the tumors for keeping Ras in the GDP-bound state. One possibility would be increased expression/activity of RasGAP, the protein that markedly accelerates the intrinsic GTPase activity of Ras (Cales *et al*., 1988). However, RasGAP activity in the samples was essentially the same in the normal mammary tissue as in the tumors (0.9 ± 0.3 pmol/min/mg protein in the normal tissue and 0.6 ± 0.2 pmol/min/mg protein in the tumors with Ki-*ras* amplification).

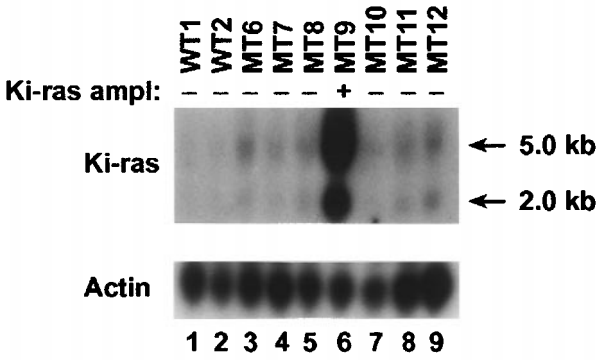

Figure 4 Representative Northern analyses of Ki-*ras* expression in SV40/Tag mammary tumors. The arrow shows the 2.0 and 5.0 kb Ki-*ras* transcripts. Lanes 1 and 2, mammary tissues of wild-type FVB/N mice (WT); lanes 3–5 and 7–9, mammary tumors (MT) without Ki-*ras* gene amplification; lane 6, mammary tumors (MT) with Ki-*ras* gene amplification

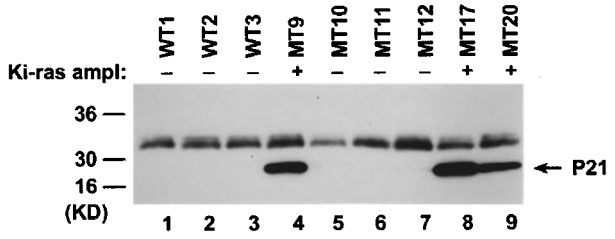

Figure 5 Representative Western analysis of Ki-*ras* protein in mammary tumors with and without Ki-*ras* gene amplification. Lanes 1–3, normal mammary tissues of wild-type FVB/N mice (MT); lanes 4 and 8–9, mammary tumors (MT) with Ki-*ras* gene amplification; lanes 5–7, mammary tumors (MT) without Ki-*ras* gene amplification. The arrow indicates the 21 kD Ras protein. The higher molecular weight band is a non-specific protein which hybridizes to the anti-mouse IgG-HRP secondary antibody (data not shown here)

Elevation MAP kinase activity

Because Ras-dependent activation of mitogen-activating protein (MAP) kinase is a major mechanism for Ras-induced proliferation and transformation (Marshall, 1995), we assessed the activation state of MAP kinase in the normal and malignant mammary tissues. We found that MAP kinase activity was increased approximately twofold in the tumors without Ki-*ras* amplification and fourfold in the tumors with Ki-*ras* amplification (Figure 6), with the increased MAP kinase activity in the tumors with Ki-*ras* amplification

Table 1 Ras-bound GTP and GDP[a]

Sample	*GTP*	*GDP*	*GTP+GDP*	*GTP / GTP+GDP* (%)
	(fmol/mg wet weight)			
WT 21	0.78	6.3	7.1	11
WT 26	0.39	7.1	7.5	5.2
WT 27	0.76	4.8	5.5	14
MT−78	0.66	60	61	1.1
MT−80	1.4	48	49	2.7
MT−83	1.8	20	21	8.6
MT+69	0.39	358	358	0.11
MT+70	1.0	564	565	0.18
MT+71	0.24	131	131	0.18

[a]Frozen mouse mammary tissue was extracted, Ras was immunoprecipitated and Ras-bound GTP and GDP were measured as described in Materials and methods. The data are expressed as fmol of GTP or GDP per mg of wet weight of the tissue and each value is the mean of duplicate measurements. WT, wild type mammary tissue, MT−, mammary tumors without Ki-*ras* amplification and MT+, mammary tumors with Ki-*ras* amplification

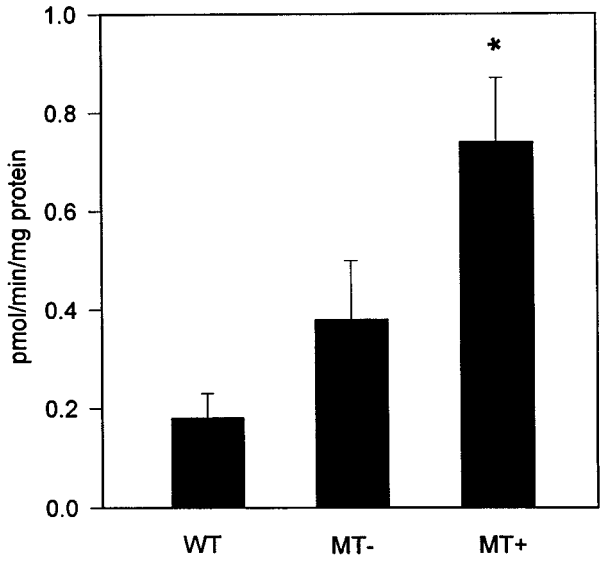

Figure 6 MAP kinase activity in mouse mammary tissues. Frozen tumor samples were extracted and MAP kinase activity was measured following phosphorylation of MBP in MAP kinase immunoprecipitates as described in Materials and methods. For each type of tissue [wild type (WT), malignant without K-*ras* amplification (MT−) and malignant with K-*ras* amplification (MT+)], the data are the means±s.d. of at least three different samples with duplicate assays having been performed on each sample. *Indicates significant difference between MT+ and WT ($P<0.05$)

being statistically significant when compared to the normal samples (as determined by a two-tailed *t* test).

Ras mutations in mammary gland tumors

To determine the frequency of Ras mutations in mammary tumors of C3(1)/SV40 Tag transgenic mice, polymerase chain reaction PCR–single strand conformation polymorphism SSCP analyses were performed in 32 mammary tumors isolated from 4–6 month old transgenic mice. Seventeen tumors had Ki-*ras* gene amplifications whereas the other 15 did not. Analyses were performed on Exons 1 and 2 of both Ha-*ras* and Ki-*ras*, regions which contain clusters of the known transforming mutations. The results are summarized in Table 2. No activation mutation in Ki-*ras* was detected in any of the tumors examined, including samples with or without Ki-*ras* amplification. Three out of thirty two (9.4%) mammary tumors did have Ha-*ras* mutations at codon 12, in which GGA was transversed to GTA, resulting in an amino acid change from glycine to valine. However, none of these three Ha-*ras* mutated tumors carried an amplifiction of the Ki-*ras* gene. These data indicate that activating Ras mutations are relatively rare in mammary tumors in C3(1)/SV40 Tag mammary tumors.

ErbB2/neu/HER2 expression

Because the SV40 large T antigen has been reported to suppress the ErbB2/neu/HER2 promoter in NIH3T3 cells (Martin and Hung, 1993), we assessed expression of the ErbB2 protein in normal and malignant mammary tissues by immunohistochemical staining. We found no clear difference among the three types of tissues, although there was a trend towards increased ErbB2 expression in the tumors with Ki-*ras* amplification (data not shown).

Table 2 Ras mutations in SV40/Tag mammary tumors

Gene	*Exon*	*Mutation*[a]	*Codon*	*Change* *Sequence*	*Amino acid*
Ha-*ras*	1	3/32	12	GGA▶GTA	Gly▶Val
	2	0/32			
Ki-*ras*	1	0/32			
	2	0/32			

[a]Ha-*ras* and Ki-*ras* mutations were examined in mammary tumors by SSCP as described in Materials and methods. Results are expressed as the number of mutated tumors in total samples examined

Discussion

CGH is a powerful molecular cytogenetic method that is capable of detecting and mapping relative DNA sequence copy number along the chromosome. We have used this method to screen for the genetic changes associated with mammary gland tumorigenesis in C3(1)/SV40 Tag transgenic mice, a model with well defined multi-stage progression of both mammary and prostate carcinomas. In this model, preneoplastic lesions develop after 8 weeks of age and invasive adenocarcinomas develop after 16 weeks of age.

CGH identified an amplification at the distal region of chromosome 6q which includes a known proto-oncogene, Ki-*ras*. Here we report that the Ki-*ras* gene is amplified selectively up to 40-fold in mammary tumors of C3(1)/SV40 Tag transgenic mice, with corresponding elevations of both Ki-*ras* RNA and protein expression. Our results indicate that the amplification of Ki-*ras* does not occur at early stages of tumor development, but occurs in the more advanced stages of tumor progression.

The enhanced expression of cellular oncogenes as a consequence of gene amplification has been described for many oncogenes in many kinds of tumors (Aldaz *et al*., 1993; Garcia *et al*., 1989; Jenkins *et al*., 1997; Schwab *et al*., 1983). Amplification and subsequent over-expression of oncogenes such as ErbB2/neu/HER2 and c-*myc* have been frequently reported in breast cancer (Blackwell *et al*., 1990; Mariani-Constantini *et al*., 1988; Seshadri *et al*., 1993; Slamon *et al*., 1989; Theillet *et al*., 1986; Varley *et al*., 1987). Although Ki-*ras* gene amplification has not been reported in human breast cancer, it has been observed in the Y1 cell line established from a spontaneous mouse adrenocortical tumor (Schwab *et al*., 1983). Amplification and over-expression of Ha-*ras* has also been described in rat mammary gland tumors (Aldaz *et al*., 1993). The fact that ErbB2/neu/HER2 and c-*myc* amplifications are not observed in this animal model, but are relatively frequent in human breast cancer (Blackwell *et al*., 1990; Seshadri *et al*., 1993; Slamon *et al*., 1989; Varley *et al*., 1987), suggests that alternate genetic pathways may be involved in Tag-induced mammary cancer progression in mice. Our results showing that Ki-*ras* is significantly amplified during mammary gland tumor progression in C3(1)/SV40 Tag transgenic mice, together with the demonstration by others of the important role of Ha-*ras* gene alterations during rat mammary oncogenesis (Barbacid, 1987)

suggest that perturbations in *ras* gene family members may be more frequently involved in rodent than human mammary tumorigenesis.

To determine the functional activity of the over-expressed Ki-Ras protein, Ras-bound GTP and MAP kinase activity were determined. We measured Ras-bound GTP and GDP by a relatively new enzymatic method (Guha *et al*., 1996, 1997; Scheele *et al*., 1995). This method has the advantage of assessing the absolute amounts of GTP and GDP bound to Ras. Had we used the older $^{32}PO_4$-labeling method which shows only the percent of Ras in the GTP-bound state, we might conclude that the tumors showed decreased Ras activation when in fact they have the same amount of Ras in the active GTP-bound state as normal mammary tissue. Thus, one must be aware of methodologic limitations when assessing the activation state of Ras, particularly when Ras shows increased expression. Although we found no increase in Ras in the active GTP-bound state in the tumors, with or without Ki-*ras* amplification, we did find a significant increase in MAP kinase activity in the tumors with Ki-*ras* amplification. One explanation for this observation might be that Ras may be undergoing increased GTP/GDP cycling in the tumors and that this increased cycling might lead to activation of the Ras/MAP kinase pathway known to be associated with cellular proliferation and transformation (Marshall, 1995). Thus, the observed Ki-*ras* amplification may be playing a pathogenic role in this mammary tumor model. We can not rule out that the large T antigen might induce an increase in MAP kinase activity that is independent of Ras. While this could explain the increase in MAP kinase activity in all of the tumors, it cannot account for the much greater increase in MAP kinase activity in the tumors with Ki-*ras* amplification.

Activation mutations of Ras protooncogenes have been demonstrated in the development of a variety of human cancers (Fearon and Vogelstein, 1990). It is estimated that Ras mutations are present in 10–15% of the most common forms of human neoplasia (Barbacid, 1987), including 90% of pancreatic adenocarcinomas, 50% of colon carcinomas and 20% of lung carcinomas. Ras activation mutations frequently result from single point mutations at amino acid 12 or 61 (Capon *et al*., 1983). Transversion mutations at codon 13, 59 or 63 can also activate the proto-oncogene (Fasano *et al*., 1984). The most frequently activated Ras oncogene is Ki-*ras* with a point mutation in codon 12 or 61. Although Ras mutations are not very common in human breast cancer (Garcia *et al*., 1989; Prosperi *et al*., 1990; Theillet *et al*., 1986), they have been strongly implicated in the etiology of mammary adenocarcinoma in many rat carcinogenesis studies (Barbacid, 1987). Our screen for Ras family gene mutations by PCR–SSCP detected no mutations for Ki-*ras* and low levels (9%) of Ha-*ras* mutations at codon 12. These data suggest that mutations of Ras gene family members play a minor role in C3(1)/SV40 Tag mammary oncogenesis.

The importance of the elevated levels of Ras proteins in tumor progression has been documented in other systems (Aldaz *et al*., 1993; Czerniak *et al*., 1989; Shekhar and Miller, 1995). The relationship of cell growth and metastases to enhanced expression of normal Ras proteins has been demonstrated in both cell lines and in human breast cancers (Czerniak *et al*., 1989; Egan *et al*., 1987; Shekhar and Miller, 1995). For example, a quantitative correlation between Ras expression and experimental metastases has been shown in Ras transfected 10T1/2 cells (Egan *et al*., 1987). Studies of human breast cancers show that Ras protein levels are significantly higher in cancer cells than in epithelial cells of control specimens and that higher levels of Ras p21 are associated with axillary lymph node metastases (Czerniak *et al*., 1989). Increasing dosage of the activated Ha-*ras* gene via gene amplification is also reported to be important in rat mammary tumor progression and may be associated with the acquisition of the hormone-independent phenotype (Aldaz *et al*., 1993).

Although amplification of genes other than Ki-*ras* on the telomeric region of chromosome 6 may also contribute to mammary tumor progression in this model, our study indicates that the amplification and functional over-expression of Ki-*ras* gene may be pathogenically important in tumor progression in C3(1)/SV40 Tag transgenic mice. This is supported by the fact that the overexpressed Ki-Ras protein is associated with an elevation in MAP kinase activity and that the degree of amplification correlates with more advanced stages of tumor progression. Furthermore, mammary tumor cell lines isolated from C3(1)/SV40 Tag transgenic mice carrying Ki-*ras* amplifications appear to be phenotypically more aggressive. The mammary tumor cell lines bearing Ki-*ras* amplification displayed earlier onset of subcutaneous tumor growth in nude mice, as compared to similar cell lines without the Ki-*ras* amplification (C Jorcyk, unpublished data). Further studies including comparison of histological differences between tumors with or without Ki-*ras* amplification, analysis of functional effects of Ras inhibitors as well as genetic manipulations such as Ki-*ras* knock out on tumor growth are currently being conducted to better define the roles of Ki-*ras* amplifications during mammary tumor progression in this transgenic mouse model.

Materials and methods

SV40/Tag transgenic mice

Transgenic mice carrying the C3(1)/SV40 Tag transgene have been previously described (Maroulakou *et al*., 1994). Heterozygous progeny were utilized in this study. They were maintained by breeding homozygous or heterozygous transgenic mice with wild type FVB/N mice and further identified as described (Maroulakou *et al*., 1994). All studies were performed in female mice.

Comparative Genomic Hybridization (CGH)

Genomic DNAs of normal mammary gland tissues and mammary tumors were labeled by digoxigenin-11-dUTP and Bio-16-dUTP (Boehringer Mannheim, Indianapolis, IN, USA), respectively, in a standard nick-translation reaction for 2 h. The DNase I concentration was adjusted so that the final probes were 500–1000 bp in size. CGH was performed as previously described (Ried *et al*., 1995). Briefly, equal amounts (200 ng) of differentially labeled tumor DNA and normal DNA were precipitated with 10 μg of salmon sperm DNA and 30 μg of mouse Cot1 DNA (Gibco BRL, Gaithersburg, MD, USA), resuspended in

hybridization solution (50% formamide, 2 × SSC and 10% dextran sulfate), denatured and pre-annealed. The solution was then hybridized to denatured normal mouse metaphase spreads for 3–4 days at 37°C. Biotin labeled tumor DNA was detected using avidin-FITC, the digoxigenin labeled reference DNA was detected using an anti-digoxigenin Fab fragment conjugated to rhodamine (Boehringer). Images were acquired using a cooled CCD-camera (CH250, Photometrics, Tucson, AZ, USA) connected to a Leica DMRBE microscope using fluorochrome specific filters (Chroma Technology, Brattleboro, VT, USA).

DNA extraction and Southern analyses

Mammary gland tissues and tumors were isolated from wild-type FVB/N mice and transgenic mice, following sacrifice by CO_2 asphyxiation at the indicated ages. Hyperplastic mammary tissues were obtained from 3–4 month old transgenic mice, the age when hyperplasia and nodular hyperplasia are found in the mammary glands (Shibata *et al*., 1996a). Tissues were immediately frozen in dry ice or liquid nitrogen and stored in −70°C. Genomic DNA was purified using the DNA Extraction Kit (Stratagene, La Jolla, CA, USA). Twenty micrograms of genomic DNA were digested with *Xba*I, separated by electrophoresis on 1% agarose gel, transferred to a nitrocellulose membrane and hybridized to ^{32}P-labeled probes under high stringency conditions. The mouse Ki-*ras* genomic clone, S-9-2, containing a 6.8 Kb *Sal*I fragment including exon 0 was kindly provided by Dr Taylor Jacks (MIT, Boston, MA, USA). The neu cDNA clone was from Dr Robert Weinberg (MIT, Boston, MA, USA) and pMet from Dr George Vande Woude (ABL, Frederick, MD, USA).

RNA isolation and Northern analyses

Total RNA was extracted from frozen samples using RNA STAT-60 (Tel-Test 'B', Inc., Friendswood, TX, USA) according to the manufacturer's protocol, except that tumor samples were extracted twice with RNA STAT 60. Three to five micrograms of RNA were electrophoresized through a 1% agarose/formaldehyde mini-gel and blotted with appropriate probes labeled with ^{32}P-dCTP using DNA Labeling Beads (-dCTP) (Pharmacia Biotech Inc., Piscataway, NJ, USA).

Western blot

Protein extracts were prepared according to the research protocol of Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Briefly, tissues were homogenized in RIPA buffer containing proteinase inhibitors (1 × PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 10 μg/ml PMSF), incubated at 4°C for 30 min and then cleared by centrifugation. Protein concentration was measured using the Bio-RAD Protein Assay (Bio-RAD, Hercules, CA, USA). Samples were separated on 12% Tris-glycine gels (Novex, San Diego, CA, USA) and transferred to nitrocellulose membranes. Protein blots were pre-blocked with non-fat milk, incubated with primary antibody (anti-Ki-Ras F234, Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by secondary antibody (anti-mouse IgG-HRP, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and detected by chemiluminescence (NEN Life Science Products, Boston, MA, USA).

PCR–SSCP analysis

PCR based SSCP was performed in tumor genomic DNA for the detection of mutations in Ha-*ras* and Ki-*ras* genes as previously described (Shibata *et al*., 1996b). Primers used for the detection of Ha-*ras* mutations were: exon 1, 5′-GATTGGCAGCCGCTGTAGAA-3′ and 5′-GGCAGAG-CTCACCTCTATAG-3′; exon 2, 5′-CTAAGCCTGTT-GTTTTGCAG-3′ and 5′-ATAGGTGGCTCACCTGTA-CT-3′. Primers used for examination of Ki-*ras* mutations were: exon 1, 5′-TTATTGTAAGGCCTGCTGAA-3′ and 5′-GCAGCGTTACCTCTATCGTA-3′; exon 2, 5′-TTC-TCAGGACTCCTACAGG-3′ and 5′-GATTTAGTATTA-TTTATGGC-3′. Mobility shifted bands in the SSCP analysis were cut out, reamplified, purified, subcloned (TA Cloning Kit, Invitrogen, San Diego, CA, USA) and sequenced (Taq DyeDeoxy Terminator Cycle Sequencing Kit, Applied Biosystems, Foster, CA, USA).

Measurement of Ras-bound GTP and GDP

Ras was immunoprecipitated from cell lysates as previously described (Scheele *et al*., 1995). Briefly, frozen tissue was homogenized (by Dounce homogenization) in an ice-cold Hepes-based buffer containing 1% Nonidet P-40 and protease inhibitors. The resulting suspension was shaken for 10 min at 4°C to fully lyse cells and then centrifuged at 10 000 *g* for 5 min; to the supernatant was added NaCl, sodium dodecyl sulfate and deoxycholate to final concentrations of 500 mM, 0.05 and 0.5%, respectively. Half of the sample was added to protein G agarose beads, preincubated with the rat monoclonal anti-ras antibody Y13-259 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and a rabbit secondary antibody (anti rat IgG-Fc, Cappel). The other half of the sample was added to beads preincubated with rat IgG and the secondary antibody. The samples were shaken gently for 1 h at 4°C and the immunoprecipitates were washed × 4 in a detergent-based buffer and × 2 in a Tris-based buffer. The washed immunoprecipitates were resuspended in a TrisPO_4/EDTA/dithiothreitol solution and heated to 100°C for 3 min to elute GTP and GDP from the immunoprecipitated Ras. GTP was measured by converting it to ATP using the enzyme nucleoside diphosphate kinase (NDPK) and ATP was measured by the luciferase/luciferin system. This assay is sensitive to 1 fmol of GTP and was performed as described previously (Scheele *et al*., 1995) with the following two changes. First, the sample was preincubated with all components of the system except the NDPK; this preincubation removes ATP or any other cellular component contaminating the samples which could cause light generation, and thereby lowers the background signal. Second, light emission was measured in a photon counting luminometer for 5 min.

GDP was measured by converting it to GTP using pyruvate kinase and phosphoenolpyruvate with the GTP measured as described. Because the final product is again emitted light measured in a luminometer, this assay is also sensitive to 1 fmol. The reaction mixture was incubated for 30 min at 30°C and contained in a final volume of 15 μl 50 mM glycine, pH 7.8, 10 mM DTT, 8 mM $MgSO_4$, 50 μM phosphoenolpyruvate, 3 milliunits pyruvate kinase and 5 μl of sample or GDP standard. The mixture was then incubated at 100°C for 5 min to inactivate pyruvate kinase because the enzyme can catalyze the reaction of phosphoenolpyruvate with ADP to generate ATP. We have shown by HPLC that at neutral pH less than 5% of GTP is destroyed when heated at 100°C for 10 min (Scheele *et al*., 1995). When GTP is measured in the second step the sum of GTP + GDP is determined; thus, the amount of GTP in the sample must be subtracted from the amount of GTP + GDP to yield the amount of GDP.

The amounts of GDP and GTP in the samples are determined from standard curves prepared with each set of samples and the data are expressed as fmol of GTP or GDP per wet weight of the sample or per microgram DNA or milligram protein in the cell lysate. DNA was measured by a

standard fluorescence method using the fluorescent dye bisbenzimidazole and protein was measured by the Bradford method.

Measurement of RasGAP activity

RasGAP activity was measured as described (Cales *et al*., 1988). Briefly, frozen samples were extracted by sonicating in a Tris-based buffer containing protease inhibitors and the extracts were centrifuged at 10 000 *g* for 10 min. To the supernatants were added purified recombinant Ras (CalBiochem, Cambridge, MA, USA) which had been previously loaded with [α-^{32}P]GTP and the samples were incubated for 10 min at 37°C. The reaction was stopped by adding a large excess of an ice-cold buffer containing 0.5% deoxycholate, 0.05% SDS, 350 mM NaCl and Ras was immunoprecipitated from the samples by adding protein G agarose beads coated with antibody Y13-259. GTP and GDP bound to the immunoprecipitated ras were eluted as previously described and were separated by thin layer chromatography on polyethyleneimine plates eluted with 1.2 M ammonium formate/0.8 M HCl. The percentage of Ras in the GTP-bound state was calculated (GTP over GTP + GDP) and compared to a blank lacking cell extract: the amount of reduction in Ras-bound GTP is attributable to RasGAP activity. The data are expressed as pmol/min/mg protein and the assay was linear with time and protein concentration.

Measurement of MAP kinase activity

Mitogen-activated protein (MAP) kinase activity was measured as described following phosphorylation of myelin basic protein (MBP) in MAP kinase immunoprecipitates (Whitehurst *et al*., 1995). Briefly, frozen tissue samples were extracted as described for immunoprecipitating Ras and a rabbit polyclonal anti-MAP kinase antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the cell lysates along with protein G agarose beads. After a 1 h incubation, the resulting MAP kinase immunoprecipitates were washed and then incubated with MBP and [γ-^{32}P]ATP for 10 min at 30°C. The reaction was stopped by spotting an aliquot of the mix on P81 ion exchange chromatography paper and the papers were washed in dilute phosphoric acid. The papers were dried and bound radioactivity representing phosphorylated MBP was measured in a liquid scintillation counter. The data are expressed as pmol/min/mg protein and the assay was linear with time and protein concentration.

Immunohistochemistry

Immunohistochemistry was performed on frozen sections according to standard procedures using an immunostain kit (Zymed Laboratories Inc., South San Francisco, CA, USA) with diaminobenzidine as the peroxidase substrate and hematoxylin as a counterstain. A rabbit anti-human ErbB-2 polyclonal antibody with cross-reactivity to mouse (Dako Co., Carpinteria, CA, USA) was used.

Abbreviations

C3(1), rat prostatic steroid binding protein gene; CGH, comparative genomic hybridization; kb, kilobase; kD, kilodalton; MBP, myelin basic protein; MAP, mitogen-activated protein; NDPK, nucleoside diphosphate kinase; SSCP, single-strand conformational polymorphism; Tag, SV40 large T-antigen

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References

Aldaz CM, Gollahon LS and Chen A. (1993). *Cancer Res.,* **53,** 5339 – 5344.

Barbacid M. (1987). *Ann. Rev. Biochem.,* **56,** 779 – 827.

Blackwell TK, Kretzner L, Blackwood EM, Eisenman RN and Weintraub H. (1990). *Science,* **250,** 1149 – 1151.

Cales C, Hancock JF, Marshall CJ and Hall A. (1988). *Nature,* **332,** 548 – 551.

Capon DJ, Seeburg PH, McGrath JP, Hayflick JS, Edman U, Levinson AD and Goeddel DV. (1983). *Nature,* **304,** 507 – 513.

Christofori G and Hanahan D. (1994). *Cancer Biol.,* **5,** 3 – 12.

Czerniak B, Chen R, Tuziak T, Markiewski M, Kram A, Devilee Gorczyca W, Deitch D, Herz F and Koss LG. (1989). *Cancer,* **63,** 2008 – 2013.

Devilee P and Cornelisse CJ. (1994). *Biochimica et Biophysica Acta,* **1198,** 113 – 130.

Egan SE, McClarty GA, Jarolim L, Wright JA, Spiro I, Hagger G and Greenberg AH. (1987). *Mol. Cell. Biol.,* **7,** 830 – 837.

Fasano O, Aldrich T, Tamanoi F, Taparowsky E, Furth M and Wigler M. (1984). *Proc. Natl. Acad. Sci. USA,* **81,** 4008 – 4012.

Fearon ER and Vogelstein B. (1990). *Cell,* **61,** 759 – 767.

Garcia I, Dietrich P-Y, Aapro M, Vauthier G, Vadas L and Engel E. (1989). *Cancer Res.,* **49,** 6675 – 6679.

Guha A, Feldkamp MM, Lau N, Boss GR and Pawson A. (1997). *Oncogene,* **15,** 2755 – 2765.

Guha A, Lau N, Huvar I, Gutmann D, Provias J, Pawson T and Boss G. (1996). *Oncogene,* **12,** 507 – 513.

Jenkins RB, Qian J, Lieber MM and Bostwick DG. (1997). *Cancer Res.,* **57,** 524 – 531.

Jones KA, Brown MA and Solomon E. (1995). *Cancer Surv.,* **25,** 315 – 334.

Mariani-Constantini R, Escot C, Theillet C, Gentile A, Merlo G, Lidereau R and Callahan R. (1988). *Cancer Res.,* **48,** 199 – 205.

Maroulakou IG, Anver M, Garrett L and Green JE. (1994). *Proc. Natl. Acad. Sci. USA,* **91,** 11236 – 11240.

Marshall MS. (1995). *FASEB J.,* **9,** 1311 – 1318.

Matin A and Hung M-C. (1993). *Cell Growth Different.,* **4,** 1051 – 1056.

Prosperi M-T, Dupre G, Lidereau R and Goubin G. (1990). *Cancer Letts.,* **51,** 169 – 174.

Ried T, Just KE, Holtgreve-Grez H, du Manoir S, Speicher MR, Schröck E, Latham C, Blegen H, Zetterberg A, Cremer T and Auer G. (1995). *Cancer Res.,* **55,** 5415 – 5423.

Scheele JS, Rhee JM and Boss GR. (1995). *Proc. Natl. Acad. Sci. USA,* **92,** 1097 – 1100.

Schwab M, Alitalo K, Varmus HE, Bishop JM and George D. (1983). *Nature,* **303,** 497 – 501.

Seshadri R, Firgaira FA, Horsfall DJ, McCaul K, Setlur V and Kitchen P. (1993). *J. Clin. Oncol.,* **11,** 1936 – 1942.

Shekhar PVM and Miller FR. (1995). *Invas. Metast.,* **14,** 27 – 37.

Shibata M-A, Maroulakou IG, Jorcyk CL, Gold LG, Ward JM and Green JE. (1996a). *Cancer Res.,* **56,** 2998 – 3003.

Shibata M-A, Ward JM, Devor DE, Liu M-L and Green JE. (1996b). *Cancer Res.,* **56,** 4894 – 4903.

Slamon DJ, Godolphin W and Jones LA. (1989). *Science,* **244,** 707 – 710.

Theillet C, Lidereau R, Escot C, Hutzell P, Brunet M, Gest J, Schlom J and Callahan R. (1986). *Cancer Res.,* **46,** 4776–4781.

Varley JM, Swallow JE, Brammar WJ, Whittaker JL and Walker RA. (1987). *Oncogene,* **1,** 423–430.

Whitehurst CE, Owaki H, Bruder JT, Rapp UR and Gepper TD. (1995). *J. Biol. Chem.,* **270,** 5594–5599.

Wooster R, Bignell G, Lancaster J *et al*. (1995). *Nature,* **378,** 789–792.